

# Detection of Polyclonality among Clinical Isolates from Prosthetic Joint Infections

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**Prosthetic joint infection (PJI) is an increasingly important health concern in the Western world due to the rising number of joint arthroplasties. Although most infections are considered to be monomicrobial, the introduction of sonication procedures has led to an increase in the detection of polymicrobial infections. To date, no published studies have investigated the presence of different clones of the same species in the infected patient. The objective of this study was to analyze whether the phenomenon of polyclonality, or the appearance of different clones in the same sample, occurs in PJI. Bacteria isolated by sonication of the retrieved implant from patients with theoretically monomicrobial PJI were included in the study. Two techniques (random amplified polymorphic DNA [RAPD] and matrix-assisted laser desorption ionization–time of flight [MALDI-TOF] mass spectrometry) were used to determine the presence of several clones in the same sample. Results were analyzed to determine bacterial species and infection type (acute versus chronic). RAPD showed a predominance of polyclonal cases (16 of 19). However, when performing the analysis with MALDI-TOF, all cases were shown to be polyclonal. We were unable to establish any relationship between the two methodologies. Polyclonality is a common phenomenon in acute and chronic PJI. Further studies are needed to establish the potential implications of this phenomenon on patient outcomes.**

Prosthetic joint replacement, or arthroplasty, is a surgical procedure that has improved the quality of life for many people around the world, providing pain relief and improved functionality to limbs (1–3). However, 10% of all patients who undergo this operation develop complications at some point in their lives; although it is not the most common, infection is one of the most significant of these complications, having an incidence of 1% to 3% (1, 3, 4). The microorganisms that cause most cases of prosthetic joint infection (PJI) are those belonging to the genus *Staphylococcus* (60% of cases), of which infections caused by *Staphylococcus aureus* constitute 25%. Gram-negative organisms (*Enterobacteriaceae*, *Pseudomonas aeruginosa*, and other species) represent a smaller proportion of cases (10%) (1, 2, 5). Notably, up to 20% to 25% of PJIs are polymicrobial infections (1–3).

When the growth conditions of the bacteria causing PJI become hostile, as may occur during antibiotic therapy, a coping mechanism known as spontaneous random hypermutation occurs as the bacteria attempt to overcome the unfavorable environment (6). This results in the development of polyclonality or the appearance of different clones in the same sample or environment (6). Polyclonality can also occur when the patient is infected with different clones from the same section of skin, which may occur either during surgery or afterwards and is likely a more common phenomenon (7). Several reports have found polyclonality in monomicrobial infections among isolates of small-colony variant staphylococci, which present phenotypically different colonies (8–10), but this phenomenon may also happen among phenotypically identical colonies.

The main objective of this study was to determine whether polyclonality can be detected in bacterial isolates from patients with apparently monomicrobial PJI. In addition, we aimed to compare the results obtained using the matrix-assisted laser desorption ionization–time of flight (MALDI-TOF) mass spectrometry technique with those obtained from random amplified polymorphic DNA (RAPD).

## MATERIALS AND METHODS

Isolates from 19 consecutive PJIs were included in the study. We selected those consecutive species from samples obtained from PJIs diagnosed from December 2011 to December 2013 that belonged to patients who fulfilled the following criteria.

Patients were diagnosed as having PJI according to internationally established criteria (11). Clinical patient data were analyzed and classified as acute, delayed/chronic, or hematogenous PJI according to the aforementioned criteria (11). Of these patients, only those with sonicated implants that had colony counts of >10,000 CFU/ml and had positive cultures from other periprosthetic samples according to the criteria described by Atkins et al. (12) were selected for the study. Only apparently monomicrobial infections were included. All joint prostheses were sonicated using a previously described protocol (13, 14). Twenty colonies from the same species were subcultured and frozen at –80°C until further experiments were performed. Although in most cases colonies were apparently identical to each other, one of the infections presented colonies with different morphologies (small-colony variants *S. aureus* strain). Therefore, colonies were randomly selected, except in this case, where morphologically different colonies were chosen.

Antimicrobial susceptibility testing of all isolates was performed by a disc-plate assay according to CLSI (15) procedures using a turbidimeter (DensiChek Plus; bioMérieux, Marcy l’Etoile, France) to achieve a 0.5 McFarland standard turbidity. A difference between isolates was considered for a difference in the inhibition zone diameter of >5 mm. The tested antibiotics were penicillin, cefoxitin, gentamicin, levofloxacin, vancomy-

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TABLE 1 Name and sequence of the primers used in this study

Species	Primer	Sequence (5' to 3')	Reference
<i>Enterobacter cloacae</i>	ECLC-1	GGTGCGGGAA	Clementino et al. (43)
	ECLC-2	GTTTCGCTCC	
	ECLC-3	GTAGACCCGT	
<i>Pseudomonas aeruginosa</i>	PSAE-1	ACGGCCGACC	Mahenthiralingan et al. (44)
	PSAE-2	GCTGGGCGGA	
	PSAE-3	GCCCGAGCGG	
<i>Klebsiella pneumoniae</i>	F-4	GGTATCAGG	Brise and Verhoef, (45) Ashayeri-Panah et al. (46)
	AP-4	TCACGATGCA	
	A-10	GTGATCGCAT	
<i>Sphingomonas paucimobilis</i>	OPA-5	AGGGGTCTTG	Perola et al., (47)
	OPB-10	CTGCTGGAC	
	M-13	TTATGTAAAACGACGCCAGT	Hsueh et al., (48)
<i>Staphylococcus aureus</i>	OLP-6	GAGGAAGAG	Reinoso et al., (49)
<i>Staphylococcus epidermidis</i>	OLP-11	ACGATGAGCC	
<i>Staphylococcus lugdunensis</i>	OLP-13	ACCGCTGCT	

cin, co-trimoxazole, and erythromycin for gram-positive bacteria; ampicillin, amoxicillin-clavulanic acid, cefuroxime, ceftriaxone, ceftazidime, imipenem, meropenem, ertapenem, levofloxacin, co-trimoxazole, fosfomycin, gentamicin, and amikacin for *Enterobacteriaceae*; and piperacillin-tazobactam, ceftazidime, cefepime, aztreonam, imipenem, meropenem, doripenem, colistin, co-trimoxazole, doxycycline, gentamicin, amikacin, norfloxacin, and ciprofloxacin for nonfermenter Gram-negative rods (all discs from bioMérieux, Marcy l'Etoile, France). Major differences were considered when a change in the interpretation (i.e., from susceptible to resistant) was detected.

Clinical charts of the patients were retrospectively reviewed in order to obtain clinically relevant data using a predefined protocol. The criteria used for the evaluation were those defined by the Infectious Diseases Society of America (IDSA) (11). The study was approved by the Ethics in Research Committee of our institution (reference number EO 04/2015\_FJD).

**MALDI-TOF.** One loop from a pure culture was placed on a specific carrier and was then mixed with a suitable matrix (1  $\mu$ l of  $\alpha$ -cyano-4-hydroxycinnamic acid [CHCA Vitek-MS; bioMérieux, Marcy l'Etoile, France]). After drying the mixture at room temperature, the slides were inserted into a mass spectrometer (MS) (Vitek 3.2.0 to 5; bioMérieux, Marcy l'Etoile, France). The software (Vitek acquisition station MS) processed the registered signal, resulting in a spectrum of intensity versus mass in daltons (Da).

**RAPD.** Bacteria were inoculated onto Trypticase soy 5% sheep blood agar (bioMérieux, Marcy l'Etoile, France) for 24 h at an atmosphere of 5% CO<sub>2</sub> and a temperature of 37°C. The purity of each culture was checked, and then all of the biomass from each agar plate was suspended in 500  $\mu$ l of sterile distilled water. Then, samples were heated to 95°C in a thermoblock (FB15101 dry bath; Fisher Scientific, Madrid, Spain) for 30 min. Samples were then centrifuged for 5 min at 14,000 rpm, and 450  $\mu$ l of supernatant was retained. *S. aureus* DNA was extracted using the easyMag 2.0 automated DNA extractor (bioMérieux, Marcy l'Etoile, France).

Subsequently, DNA was quantified with the NanoDrop ND-1000 spectrophotometer (Thermo Scientific, Madrid, Spain), and the samples were then adjusted to a final concentration of 100 ng/ $\mu$ l DNA.

Primers used for RAPD analysis were selected from the literature (Table 1). Three different primers were used for each bacterial species. For microorganisms belonging to the genus *Staphylococcus*, common primers were used for all species.

In order to perform the amplification, a master mixture containing 19  $\mu$ l of DNA-free water and 1  $\mu$ l of primer was added to PuReTaq Ready-

To-Go PCR beads (GE Healthcare, Madrid, Spain) along with 5  $\mu$ l of the sample DNA. This was subjected to a program consisting of 39 cycles at 94°C for 1 min, 36°C for 1 min, and 72°C for 2 min. After completing all cycles, a final period of 10 min at 72°C was performed. For the genetic material extracted from staphylococci, the amplification program was initially 5 min at 94°C followed by 39 cycles of 93°C for 1 min, 37°C for 30 s, and 72°C for 1 min. This program concluded with 8 min at 72°C.

Electrophoresis was performed in a 3% agarose gel (Agarose Basic; AppliChem GmbH, Germany) to which 5  $\mu$ l of ethidium bromide was added. To prepare the gel and to immerse it in the electrophoresis tank, 1 $\times$  Tris-borate-EDTA (TBE) buffer was used. Twenty microliters of the mixture was loaded into the wells of the gel and run for 4 to 5 h at 80 V. Gels were analyzed under UV transillumination, and the images were captured for further analysis.

**Data analysis.** The spectra obtained from the mass spectrometer (MALDI-TOF technique) and the images of the agarose gels (RAPD technique) were analyzed using BioGene software (BioGene, Kimbolton Cambs, United Kingdom), which considered strains with a homology of 95% to 100% as being identical. The identities of the profiles with 3 sets of primers were used as the criteria for monoclonality using RAPD.

Data were statistically analyzed using the EPI-INFO 3.5.4 (2012) software (CDC, Atlanta, GA, USA). To compare qualitative variables, Fisher's exact test was used.

## RESULTS

During the study period, 86 culture-positive PJI were diagnosed (39 acute, 7 hematogenous, and 40 chronic/delayed). Among these, only 19 fulfill all of the established criteria to be included in the study. The bacterial species included were *Enterobacter cloacae*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, *Sphingomonas paucimobilis*, *S. aureus*, *Staphylococcus epidermidis*, and *Staphylococcus lugdunensis* (Table 2). Clinical characteristics of the patients and results of the microbiological studies appear in Table 3.

The RAPD assays of the 2 cases of *E. cloacae* revealed polyclonality in both, with detection of 7 different clones in each of the cases. In contrast, all clones of *P. aeruginosa* were identical in the 2 cases studied. As in the case of *E. cloacae*, RAPD analysis of *K. pneumoniae* showed the existence of 7 individual clones. Another example of polyclonality was found in the analysis of *S. paucimobilis*, with 9 different clones. Regarding analyses of *S. aureus*, the

**TABLE 2** Number and percentage of infections caused by each organism isolated for the total number of cases studied (19 cases)

Bacterial species	No. of infections	Percentage of infections
<i>Enterobacter cloacae</i>	2	10.52
<i>Pseudomonas aeruginosa</i>	2	10.52
<i>Klebsiella pneumoniae</i>	1	5.26
<i>Sphingomonas paucimobilis</i>	1	5.26
<i>Staphylococcus aureus</i>	9	47.37
<i>Staphylococcus epidermidis</i>	3	15.79
<i>Staphylococcus lugdunensis</i>	1	5.26

results were highly variable, ranging from monoclonal infections (1 case) to cases in which there were 2, 4, 5, 7, 8, 10, 11, and even 12 different clones. Analysis of *S. epidermidis* showed less variability than that of *S. aureus*, resulting in a maximum of 3 different clones (2 of the cases). The only case of *S. lugdunensis* had 4 different clones.

Based on the results obtained by RAPD, there was a clear predominance of polyclonal infections (16 of the 19 cases studied). The causal organisms of monoclonal infections were *S. aureus* (1 case) and *P. aeruginosa* (2 cases). Conversely, the analysis performed with MALDI-TOF suggested that all infections were polyclonal, including the cases that were considered to be monoclonal by RAPD. Figures 1 and 2 illustrate examples of the spectra obtained by MALDI and the corresponding dendrogram for one case of *E. cloacae*. Examples of the dendrograms obtained from RAPD images are shown in Fig. 3.

Regarding clinical data, we compared acute infections (10 cases) versus chronic/delayed infections (9 cases). Fisher's exact test revealed no significant differences when the presence of polyclonality between acute and chronic/delayed prosthetic infections was compared. Likewise, although no significant differences were found when comparing *S. aureus* and *S. epidermidis*, the sample was too small to get a reliable conclusion from those data.

## DISCUSSION

Although the etiology of prosthetic infection is diverse, several studies have shown that the most frequently isolated microorganisms are staphylococci (1–3). *S. aureus* is a commonly described microorganism in different hospital-acquired infections (16), probably because of its abundance on the skin, as in the case of *S. epidermidis* (17). Several authors have concluded that the pathogenicity of these microorganisms in implant-related infections lies in their ability to form biofilms (2, 18, 19), as these structures protect the bacteria from the immune system and also make them less susceptible to antibiotics (2, 7, 19–22). We must also not forget other species of staphylococci, such as *S. lugdunensis*, which is being increasingly recognized as the cause of severe infections (2, 23). The emergence of these organisms may be related to the presence of a gene complex with a similar order and sequence in all of the 3 aforementioned species (20). Other species studied in this work, such as *P. aeruginosa*, *E. cloacae*, *S. paucimobilis*, and *K. pneumoniae*, have also been reported to cause PJI, although less frequently than staphylococci (2, 24, 25).

The use of low-intensity ultrasound in the infected prosthesis releases the biofilm without destroying the microorganisms, thereby improving the sensitivity of conventional cultures (2, 3, 21, 22). Our study aimed to determine whether several clones of microorganisms can be found in apparently monomicrobial in-

fections. Our hypothesis rests on the “race for the surface” theory (19, 26, 27), as we think that several clones of the same bacteria can contaminate the prosthesis during the surgery and that we may only identify these bacteria through molecular techniques.

Our results support the role of RAPD typing as a useful tool for the detection of genomic polymorphisms (28). In fact, there have been studies of the strain differentiation of *S. aureus* based on this technique (16, 29). Our results show that 1 case of *S. aureus* infection was monoclonal but that 8 cases were polyclonal, with a varying number of clones between them. According to Byun et al., (16) the combination of several primers increases the ability to discriminate between strains. Ueta et al. (17) reported the detection of different genetic profiles of *S. epidermidis* isolated from the conjunctival sac of the same subject. Although the reproducibility of this technique is problematic (30), this problem can be minimized if all strains are processed simultaneously in the same gel, as we have done in our study. Moreover, the use of restrictive criteria to establish monoclonality increases the likelihood that our results closely resemble those obtained with other techniques. In addition, this technique is easy to perform for laboratories with no access to more reliable techniques, such as pulsed-field gel electrophoresis (PFGE) or complete DNA sequencing, which require the use of more specialized installations.

Nowadays, MALDI-TOF is one of the most highly valued methodologies in bacterial identification (3, 31–33). Unlike RAPD, it is based on the analysis of bacterial proteins. This is probably the main cause of the absence of monoclonal cases in our study. Protein synthesis can be quite variable from one organism to another since this process is affected by factors such as the availability of mRNA (pretranslational level) or reading effectiveness (translational level). There are also numerous inhibitors of the process of protein biosynthesis, which act by blocking events in the initiation and elongation stages. All of this increases the possibility of detecting differences between strains of the same microorganism.

Although MALDI-TOF mass spectrometry can be used as a reliable technique in the bacterial identification at the species level (34), it is not yet clear whether this method makes it possible to differentiate at the strain level (33). Some authors have stated that the discriminatory power of MALDI-TOF to distinguish different clones of *S. aureus* and *Enterococcus faecium* is insufficient compared with that of a molecular technique such as multilocus sequence typing (MLST) (34). According to these authors, it is highly unlikely that a single marker peak (the difference between 2 strains) has sufficient discriminatory power to allow the formation of clusters and may be unreliable for the identification of clonal lineages (34). These authors support their claim by explaining that MALDI-TOF only detects a bacterial subproteome in a limited range of masses and that only a selected number of proteins are ionized, which reduces the number of signals available for strain characterization. Another report supports the ability of MALDI-TOF for subtyping (35). In the study, 158 isolates of staphylococci were characterized by MALDI-TOF using specific software, ensuring 100% accuracy at the genus and species levels and thus showing a great potential for discrimination between strains. In fact, this methodology has been used to differentiate between strains of *E. coli* (36, 37). This approach and particularly the dendrogram analysis may be improved if common spectral peaks or biomarkers are omitted (38).

In our study, the results of MALDI-TOF analysis show clear

TABLE 3 Epidemiological, clinical, and microbiological data of the studied cases

Patient	Age	Sex <sup>a</sup>	Joint	Date of implant surgery (mo/day/yr)	Date of implant removal (mo/day/yr)	Microbiology of the implant			Major differences in antimicrobial susceptibility	Treatment <sup>b</sup>	Other positives samples	Number of clones obtained			Clinical outcome
						Microorganism	CFU/ml					RAPD	MALDI-TOF		
1	75	M	Knee	07/11/2013	09/20/2013	<i>S. epidermidis</i>	80,000	No	No	Vancomycin + fosfomycin	1 Synovial fluid	3	7		Good (24 mo)
2	70	M	Hip	17/12/2013	01/10/2014	<i>S. aureus</i>	10,000	No	No	Levofloxacin + rifampin, linezolid + rifampin	1 Wound exudate + 1 synovial fluid	1	4		Reinfection
3	69	F	Knee	11/08/2012	10/24/2013	<i>S. aureus</i>	>100,000	No	No	Rifampin + co-trimoxazole	2 Synovial fluid + periprosthetic tissue samples	4	10		Good (18 mo)
4	78	F	Hip	02/26/2013	05/31/2013	<i>S. epidermidis</i>	10,000	No	No	Vancomycin + rifampin	Periprosthetic tissue samples	3	7		Good (12 mo)
5	43	M	Hip	11/16/2012	11/29/2012	<i>E. cloacae</i>	100,000	No	No	Co-trimoxazole + levofloxacin	1 Periprosthetic tissue sample	7	2		Good (33 mo)
6	92	F	Hip	12/13/2013	01/14/2014	<i>S. aureus</i>	>100,000	No	No	Clindamycin + rifampin	1 Synovial fluid	2	8		Follow up lost after 4 mo (good until this date)
7	56	M	Knee	04/03/2012	03/19/2013	<i>S. lugdunensis</i>	>100,000	No	No	Levofloxacin + rifampin	3 Synovial fluids	4	3		Reinfection
8	70	F	Hip	12/27/2012	01/08/2013	<i>P. aeruginosa</i>	10,000	No	No	Ciprofloxacin + amikacin + cefepime	1 Synovial fluid	1	4		Good (7 mo)
9	87	F	Hip	08/09/2013	08/26/2013	<i>P. aeruginosa</i>	30,000	No	No	Ciprofloxacin + amikacin + cefepime	1 Drainage fluid, 1 hematoma, 2 subcutaneous tissue, 1 wound exudate	1	4		Good (12 mo)
10	51	M	Hip	11/13/2013	12/11/2013	SCV <sup>c</sup> <i>S. aureus</i>	10,000	Yes <sup>d</sup>	Yes <sup>d</sup>	Vancomycin + rifampin, Levofloxacin + rifampin	1 Wound exudate, 1 periprosthetic tissue sample, 1 synovial fluid	11	5		Good (18 mo)
11	82	M	Hip	12/27/2011	02/25/2014	<i>S. aureus</i>	>100,000	Yes <sup>e</sup>	Yes <sup>e</sup>	Levofloxacin + co-trimoxazole	6 Periprosthetic tissue samples and 2 wound exudates	12	5		Good (12 mo)
12	75	M	Knee	12/23/2013	01/16/2014	<i>S. aureus</i>	12,000	No	No	Clindamycin + rifampin, co-trimoxazole + linezolid	1 Synovial biopsy, 1 synovial fluid, 1 wound exudate	7	4		Follow up lost after 1 mo
13	44	M	Hip	06/11/2013	07/03/2013	<i>S. aureus</i>	>100,000	No	No	Levofloxacin + rifampin	1 Periprosthetic tissue sample, 1 synovial fluid, 1 wound exudate	10	2		Good (13 mo)
14	71	M	Knee	08/26/2013	09/17/2013	<i>S. aureus</i>	>100,000	No	No	Levofloxacin + rifampin	1 Periprosthetic tissue sample, 1 wound exudate, 1 subcutaneous tissue, 1 hematoma	8	5		Good (20 mo)
15	79	F	Knee	01/14/2013	04/15/2013	<i>E. cloacae</i>	50,000	No	No	Ciprofloxacin + co-trimoxazole	2 Synovial fluids, 1 periprosthetic tissue sample	7	5		Good (26 mo)
16	87	M	Knee	03/12/2013	01/30/2014	<i>S. epidermidis</i>	>100,000	No	No	Vancomycin + rifampin	1 Synovial fluid, 5 periprosthetic tissue samples	2	5		Reinfection
17	85	M	Hip	12/13/2013	01/30/2014	<i>S. aureus</i>	>100,000	No	No	Vancomycin + rifampin, co-trimoxazole + rifampin	1 Synovial fluid, 1 periprosthetic tissue sample	5	7		Good (15 mo)
18	81	F	Hip	04/25/2012	10/04/2013	<i>S. paucimobilis</i>	10,000	No	No	No	1 Periprosthetic tissue sample	9	4		Death due to other underlying diseases 1 day after surgery
19	87	F	Hip	02/27/2013	13/03/2013	<i>K. pneumoniae</i>	10,000	No	No	Amoxicillin-clavulanic, imipenem + amikacin	1 Wound exudate, 1 synovial fluid, 1 periprosthetic tissue sample	7	6		Follow up lost after 1 mo

<sup>a</sup> M, male; F, female.

<sup>b</sup> Empirical therapy during the study period was vancomycin + ceftazidime.

<sup>c</sup> SCV, small-colony variant.

<sup>d</sup> Case 10: Difference of 1 clone with gentamicin, levofloxacin, co-trimoxazole, and erythromycin. 1 clone showed inhibition zones higher than 40 mm for all antibiotics. Another clone showed major difference for doxycycline. Three clones showed major differences for co-trimoxazole and erythromycin.

<sup>e</sup> Case 11: Major difference of 2 clones with gentamicin. Two clones showed inhibition zones higher than 40 mm for cefoxitin, co-trimoxazole, and erythromycin.



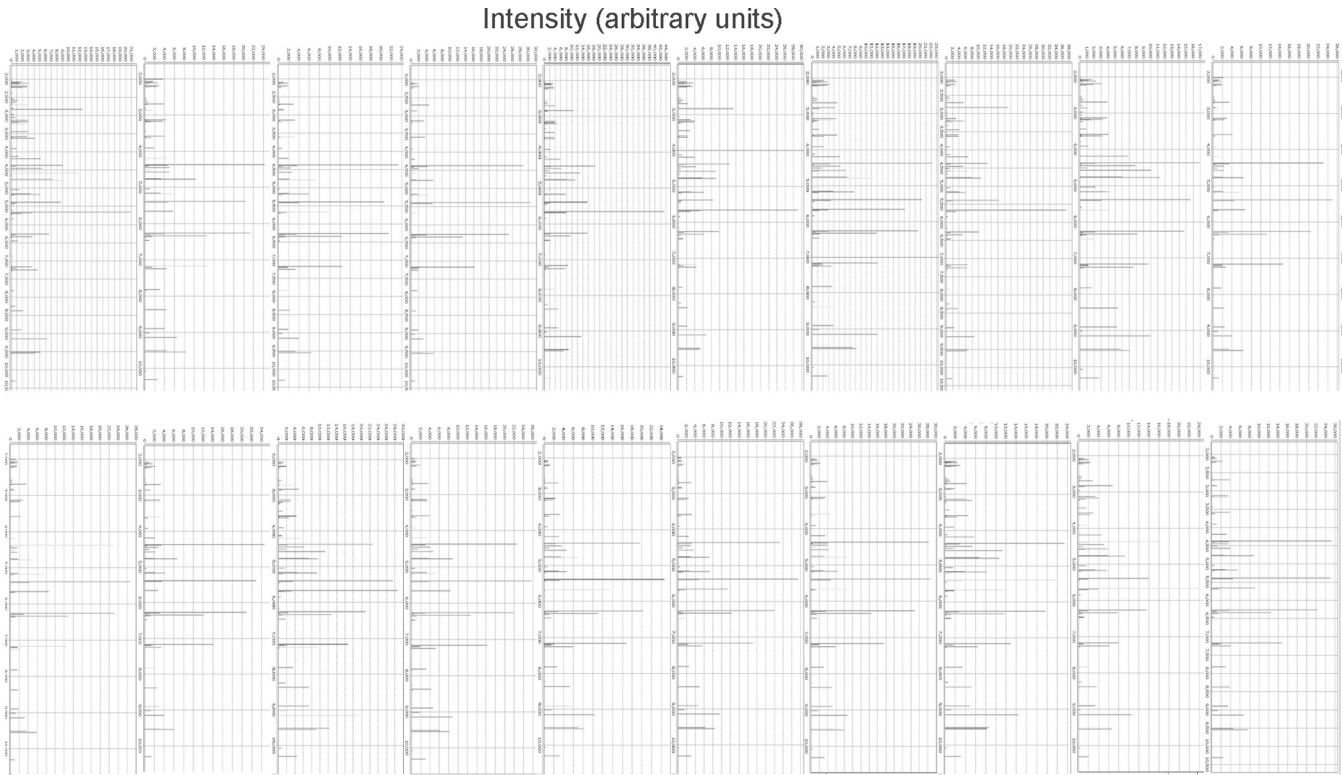


FIG 1 Example of spectra and dendrogram corresponding to case 5 (*E. cloacae*). BioGene software analyzes the spectra and considers the strains with a 95% to 100% homology to be identical. The dendrogram groups these strains with the same spectrum.

identification at the genus and species levels but do not allow for a common approach to be established to discriminate between strains, although the resulting dendrograms reveal clear differences in the clones studied. Again, the small number of cases studied and the lack of specific software to establish the clonality of isolates are important limitation of this study. Therefore, it is necessary to conduct more studies with this technique using a greater number of cases in order to homogenize the criteria for identification

and identity between strains compared with those of other widely accepted molecular techniques.

The main limitation of our report is our selection of techniques. RAPD has been claimed as a technique with low reproducibility, but we have the experience that, if strict conditions and criteria are followed, the obtained results can be considered useful for discrimination purposes (39–41). This technique also has the advantage of being easy to perform without complex equipment,

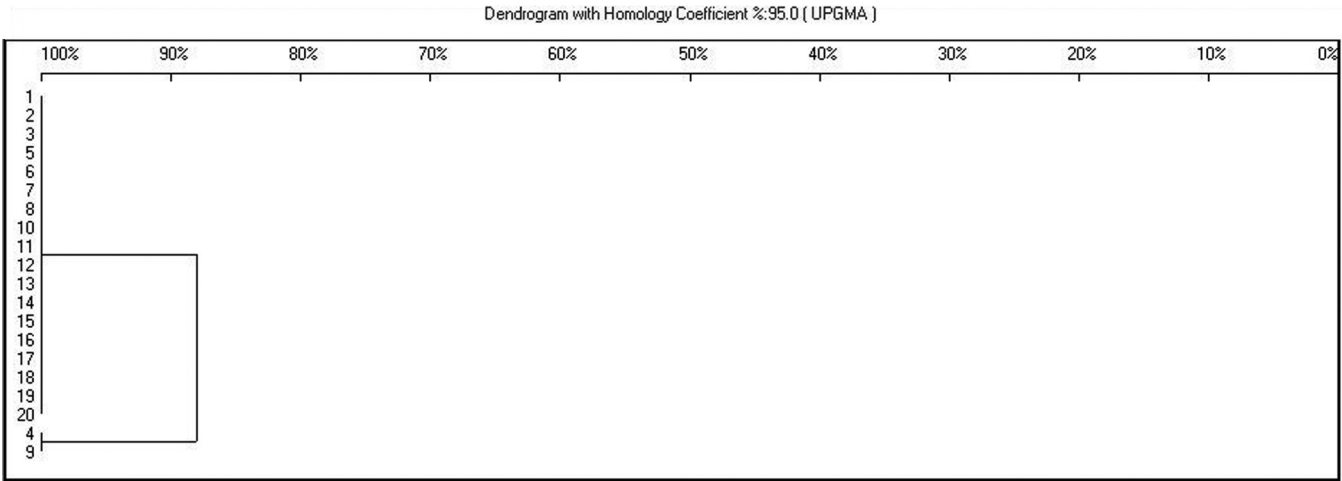


FIG 2 Example of spectra and dendrogram corresponding to case 5 (*E. cloacae*). BioGene software analyzes the spectra and considers the strains with a 95% to 100% homology to be identical. The dendrogram groups these strains with the same spectrum.

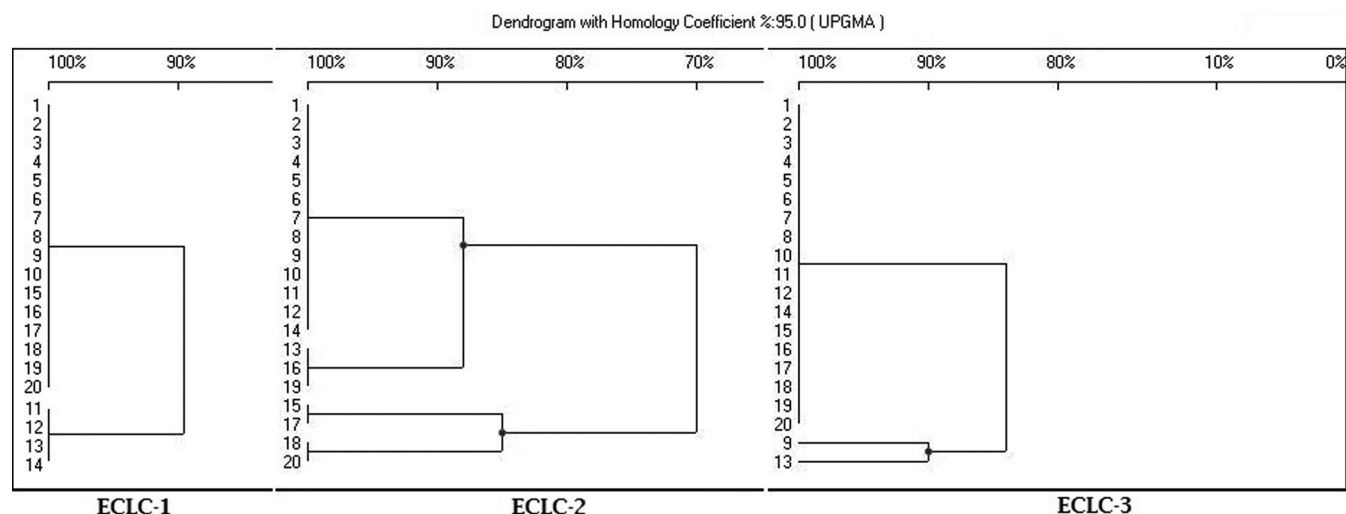


FIG 3 Dendrograms obtained with the 3 primers used for *Enterobacter cloacae* (case 5) by RAPD. BioGene software groups, for each primer (ECLC1, ECLC2, and ECLC3), strains with a 95% to 100% homology.

such as that needed for other reference techniques like PFGE or complete-genome sequencing, so it can be performed in medium-size laboratories like ours. Regarding the MALDI-TOF methodology, as recently reviewed (42), this technique lacks the proper definition of criteria for the interpretation of the results, and this limitation must be considered carefully in this research. Because of these limitations, further studies are needed to confirm or deny the results obtained by our study.

In conclusion, the RAPD technique revealed 16 cases of polyclonality among 19 cases of monomicrobial PJI. The MALDI-TOF methodology showed an even higher percentage, with all cases being polyclonal. When performing statistical analysis, no significant differences in the appearance of polyclonality were found when comparing acute and chronic prosthetic infections. No correspondence was detected between the two techniques. Further studies are needed to confirm these results and to establish the actual role of this phenomenon in patient outcomes.

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